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PP1 Phosphatase complexes: no longer undruggable enzymes.

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Abstract: Krzyzosiak et al, describe a paradigm changing scheme to identify inhibitors targeting regulatory subunits of serine/threonine PP1 phosphatases complexes. They characterize a selective R15B phosphatase regulatory subunit inhibitor termed Raphin1. This protects cells from stress, delaying neurodegeneration in a mouse model of Huntington's disease, illustrating the potential of this approach.

The ability of a cell to rapidly slow down protein synthesis following exposure to a stress is one of the fundamental responses that enables eukaryotic cells to tolerate such conditions. This increases capacity of chaperones and proteosomal machinery to clear misfolded proteins before they accumulate to dangerous levels (Donnelly et al., 2013)). Key to the ability of stress to attenuate protein synthesis, is the phosphorylation of the α-subunit of the eukaryotic translation initiation factor 2 (eIF2α) on Serine 51. There are four Ser51 eIF2α kinases (general control non-depressible (GCN2), heme-regulated inhibitor (HRI), PKR-like endoplasmic reticulum kinase (PERK) and protein kinase R (PKR)) that all phosphorylate Ser51 of eIF2α in response to different types of stress, illustrating the importance of this response pathway (Donnelly et al., 2013) (Figure 1 A).

Screens undertaken some years ago identified two compounds termed Guanabenz (Tsaytler et al., 2011) and Sephin1 (Das et al., 2015) that protected cells from otherwise lethal doses of endoplasmic reticulum stress that induces unfolding of proteins. These compounds inhibit a protein phosphatase 1 (PP1) holoenzyme (complex consisting of the PP1 catalytic

subunit and a regulatory subunit termed R15A) that acts to dephosphorylate eIF2α at Ser51 (Harding et al., 2009) and thus they exert their stress protective properties by prolonging the beneficial effects of eIF2α phosphorylation.

Cells possess another PP1 regulatory subunit termed R15B, which is 28% identical to R15A, that appears to play a more general role in dephosphorylating eIF2α (Tsaytler et al., 2011 and Das, 2015 #13) (Figure 1A). Guanabenz and Sephin-1 are selective for the R15A PP1 complex and do not significantly inhibit R15B PP1. Krzyzosiak et al reasoned that targeting the R15B:PP1 complex instead of R15A could be a better choice to obtain inhibitors that were effective against diverse stress. This was an ambitious quest, as PP1 holoenzyme complexes had never been targeted previously.

To achieve this, Krzyzosiak and co-workers reconstituted the partial R15A/PP1 and R15B-PP1 holoenzymes on a surface plasmon resonance sensor chip and undertook a compound binding screen with a small library of 69 derivatives of Ganabenz. This lead to the discovery of the compound termed Raphin1, that bound R15B-PP1 with 30-fold selectivity over the R15A-PP1 complex. When used in cells, Raphin1 increased eIF2α phosphorylation and decreased protein synthesis. These effects were lost following knock-out of the R15B subunit, confirming that the mechanism of action of Raphin1 was indeed mediated through R15B. The effects on Raphin1 on eIF2α phosphorylation and protein synthesis were transient, with recovery observed after around 10 hours. Further analysis revealed that this

was caused by marked upregulation of the R15A subunit occurring after about 2 h of Raphin1 administration. More work is needed to establish the mechanism by which cells upregulate R15A following inhibition of R15B.

Raphin1, when administered to mice at doses as low as 2mg/kg, accumulated in tissues including brain at sufficiently high concentrations to inhibit R15B and had no reported adverse effects on the animals. When Raphin1 was administered once per day to a mouse model of Huntington's disease over a 6-week period, it markedly decreased insoluble Huntingtin protein aggregates and also significantly delayed disease onset. There are currently no treatments that prevent or even delay disease progression of Huntington's and other neurodegenerative disorders caused by abnormal protein misfolding. In future work it will be vital to further explore whether Raphin1 or other R15B inhibitors have therapeutic benefit for these devastating diseases in humans.

Research into the regulation of protein phosphorylation over the last decades has focused on kinases and the examination of phosphatases has stagnated. Kinase inhibitors have become one of the pharmaceutical companies favored targets, with at least 48 clinically approved compounds (http://www.kinase-screen.mrc.ac.uk/phosphorylation-ubiquitylation-drug-discovery) and many more are in clinical development (Ferguson and Gray, 2018). The only drugs that target a protein phosphatase complex are the macrolide FK506 and its derivatives, immunosuppressive agents developed over 20 years ago. These bind to the immunophilin FKBP12, and suppress activity the calcineurin

(protein phosphatase 3) complex, thereby blocking activation of the transcription factor NFATc (nuclear factor of activated T cell cytoplasmic) in T cells (Rusnak and Mertz, 2000). To our knowledge only a single phosphatase inhibitor termed LB100, that targets PP2A, is currently in early phase clinical trials, for treatment of recurrent glioblastoma. Selective allosteric inhibitors of a variety of tyrosine phosphatases including PTP1B (Protein tyrosine phosphatase 1B-obesity and associated co-morbidities), SHP2 (the tyrosine phosphatase produced by the oncogene PTPN11-oncology) and SHP1 (produced by the PTPN6 gene oncology and immune disorders) have been elaborated, but research is still at the preclinical stage (He et al., 2014).

Drug discovery efforts targeting the catalytic subunits of PP1 were quickly abandoned in the 1990's as natural compounds including microcystin and calyculin A, that inhibited all PP1 holoenzymes were found to be highly toxic (Cohen, 2002). It was assumed infeasible to target specific PP1 holoenzymes. The work performed by Krzyzosiak et al, provides much-needed ammunition that crafting selective inhibitors targeting PP1 holoenzymes is indeed achievable. Raphin-1 binding to R15B induces a conformational change resulting in it becoming targeted for p97 and proteasome mediated degradation *in vivo* (KRZYZOSIAK Ref). However, there are other mechanisms of action that could be considered for developing inhibitors of PP1 holoenzymes in future studies, increasing opportunity for success (Figure 1 B). These include blocking the PP1 regulatory subunit from accessing its physiological substrate or inducing disassembly of regulatory and catalytic subunits. There may also be other functionally critical pockets

on certain PP1 holoenzymes that could be exploited (Fig 1B). In future work, we would advocate incorporating unbiased quantitative total and phosphoproteomic into the workflow (that was not utilized in the Krzyzosiak et al study), to better characterize the *in vivo* specificity of identified PP1 holoenzyme inhibitors.

The Krzyzosiak study represents a major step forward, providing the strongest evidence to date that PP1 holoenzymes are indeed druggable. The research validates R15B as a promising target to slow progression of a range of devastating neurodegenerative conditions. Perhaps the greatest challenge going forward will be to define which PP1 holoenzyme complexes should be targeted to treat specific diseases (Ferreira et al., 2018) as the physiological roles of the vast majority of these enzymes, of which there is over 200, is unknown. Fundamental mechanistic research in this neglected field of phosphatase biology is urgently needed. Defining the key set of physiological substrates that individual PP1 holoenzymes act upon, would significantly contribute to the growing armory of approaches that can be deployed to modulate protein phosphorylation for treatment of disease.

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Figure legend

Protein phosphatase holocomplexes are possible drug targets.

A) Eukaryotic translation initiation factor 2 (eIF2α) is phosphorylated at Ser51 during stress response to slow down translation. Four different kinases (PERK, PKR, CGN2 and HRI) can phosphorylate eIF2α depending on type of stress. Two different but related protein phosphatase 1 (PP1) holoenzymes (complex consisting of the PP1 catalytic subunit (PP1c) and a regulatory subunit termed R15A or R15B) counteract these phosphorylations.

The newly discovered drug Raphin1 binds selectively to the regulatory subunit R15B, causing a conformational change to the protein structure that ultimately leads to degradation of the subunit, thus inactivating the phosphatase complex.

B) Alternative strategies could be used to target the PP1 holoenzymes (PP1c + R). Inhibitors (I) can be designed: 1) to block the binding of the regulatory subunit (R) with the substrate (S) (substrate recognition pocket); 2) to block the binding between PP1c and the regulatory subunit (PP1 binding pocket); 3) to interfere with other essential domains of the regulatory subunit (other functional pocket).

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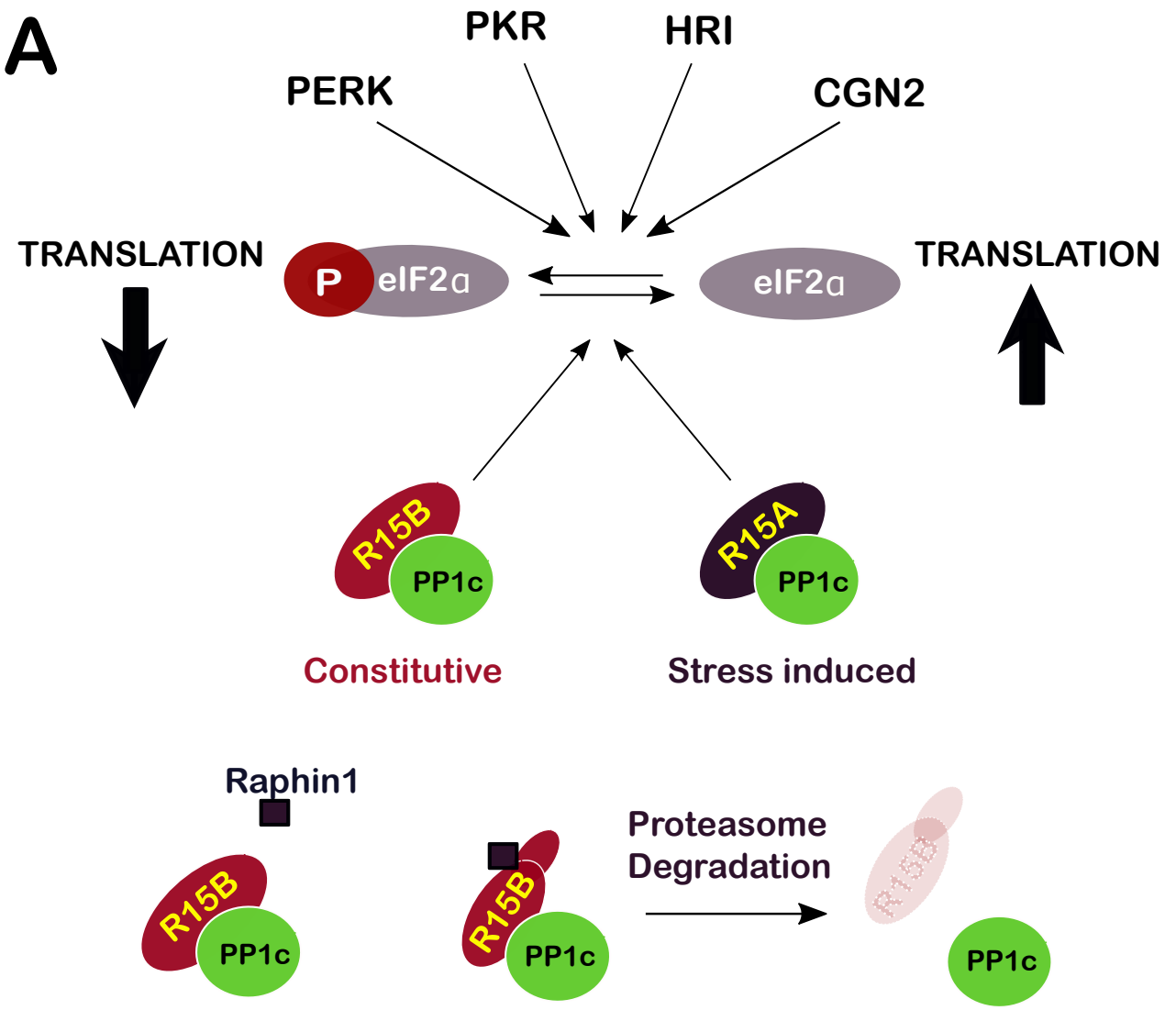
A
PKR
HRI
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TRANSLATION
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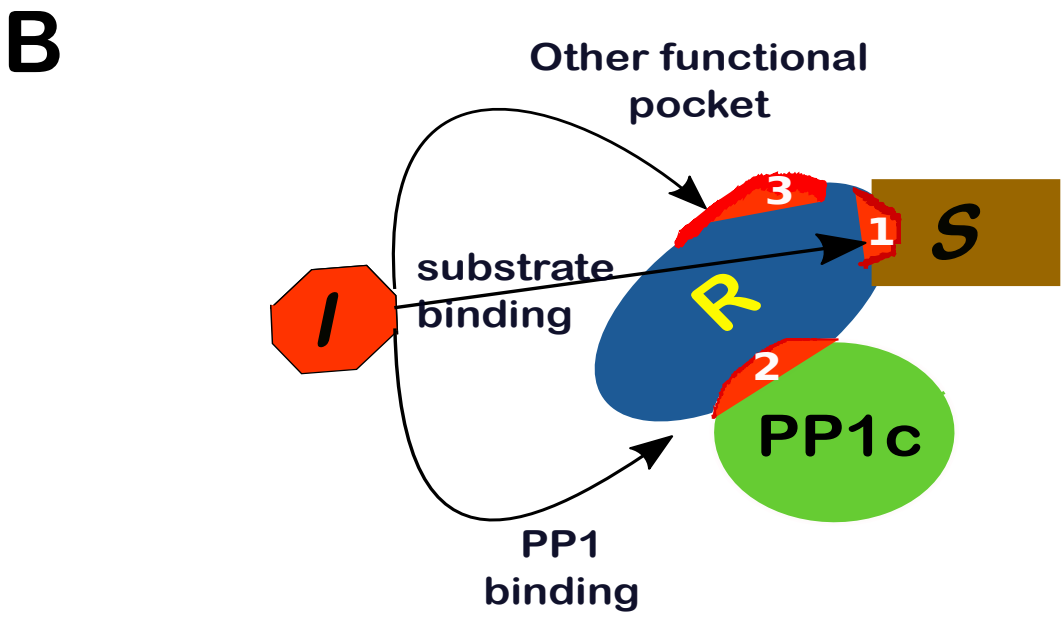
eIF2α
TRANSLATION
R15B
PP1c
R15A
PP1c
Constitutive
Stress induced
Raphin1
R15B
PP1c
R15B
PP1c
Proteasome
Degradation
PP1c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